Signaling pathways mediating gastrointestinal smooth muscle contraction and MLC$_{20}$ phosphorylation by motilin receptors

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Huang, Jiean, Huiping Zhou, Sunila Mahavadi, Wimolpak Sriwai, Vijay Lyall, and Karnam S. Murthy. Signaling pathways mediating gastrointestinal smooth muscle contraction and MLC$_{20}$ phosphorylation by motilin receptors. Am J Physiol Gastrointest Liver Physiol 288: G23–G31, 2005; doi:10.1152/ajpgi.00305.2004.—The signaling cascades initiated by motilin receptors in gastric and intestinal smooth muscle cells were characterized. Motilin bound with high affinity (IC$_{50}$, 0.7 ± 0.2 nM) to receptors on smooth muscle cells; the receptors were rapidly internalized via G protein-co coupled receptor kinase 2 (GRK2). Motilin selectively activated Go and G13, stimulated Go$_{q}$-dependent phosphoinositide (PI) hydrolysis and 1,4,5-trisphosphate (IP$_3$)-dependent Ca$^{2+}$ release, and increased cytosolic free Ca$^{2+}$. PI hydrolysis was blocked by expression of Go$_{q}$ mimigene and augmented by overexpression of dominant negative RGS4 (N88S) or GRK2 (K220R). Motilin induced a biphasic, concentration-dependent contraction (EC$_{50}$ = 1.0 ± 0.2 nM), consisting of an initial peak followed by a sustained contraction. The initial Ca$^{2+}$-dependent contraction and myosin light chain (MLC$_{20}$) phosphorylation were inhibited by the PLC inhibitor U-73122 and the ML kinase inhibitor ML-9 but were not affected by the Rho kinase inhibitor Y27632 or the PKC inhibitor bisindolylmaleimide. Sustained contraction and MLC$_{20}$ phosphorylation were RhoA dependent and mediated by two downstream messengers: PKC and Rho kinase. The latter was partly inhibited by expression of Go$_{q}$ or Go$_{13}$ mimigene and abolished by coexpression of both mimigenes. Sustained contraction and MLC$_{20}$ phosphorylation were partly inhibited by Y27632 and bisindolylmaleimide and abolished by a combination of both inhibitors. The inhibition reflected phosphorylation of both PLC and phosphatase inhibitors: CPI-17 via PKC and MYPT1 via Rho kinase. We conclude that motilin initiates a Go$_{q}$-mediated cascade involving Ca$^{2+}$/calmodulin activation of MLC kinase and transient MLC$_{20}$ phosphorylation and contraction as well as a sustained Go$_{q}$- and Go$_{13}$-mediated, RhoA-dependent cascade involving phosphorylation of CPI-17 by PKC and MYPT1 by Rho kinase, leading to inhibition of PLC phosphatase and sustained MLC$_{20}$ phosphorylation and contraction.

interdigestive gut hormone; Rho kinase; G protein signaling

MOTILIN, a 22-AMINO ACID PEPTIDE released from endocrine cells of the upper small intestine, is the only interdigestive gut hormone (1). Motilin is released in cycles that coincide with phase III of the migrating myoelectric complex, a 5- to 10-minute period of intense, migrating rhythmic motor activity that typically arises in the gastric antrum (70%) or duodenum (30%) (3, 10, 29, 37). Immunoneutralization of circulating motilin suppresses phase III motor activity, and infusion of motilin in concentrations that mimic circulating levels induces premature phase III activity (11, 14). The latter is retained in autotransplanted gastric pouches, implying that it is largely independent of extrinsic neural input but is suppressed by atropine, suggesting that motilin acts by activating myenteric cholinergic neurons either directly or via interneurons (36). In vivo experiments using close intra-arterial injection of motilin suggest in effect that neural motilin receptors may not be located directly on cholinergic motoneurons (30).

Numerous studies have demonstrated the presence of motilin receptors on smooth muscle cells, which appear to facilitate or augment nerve-mediated effects of motilin (4, 6, 18, 27, 28, 34). Radioligand binding studies on gastric and intestinal membranes enriched with smooth muscle markers (5'-nucleotidase) provide evidence for the presence of motilin receptors on smooth muscle cells (3, 8, 36). Motilin activates L-type Ca$^{2+}$ channels in dispersed human and canine intestinal muscle cells (5, 16) and enhances carbachol-induced cationic current in dispersed duodenal muscle cells (38). Motilin and related motilides such as erythromycin induce contraction of guinea pig, rat, and human gastric muscle cells, canine jejunal muscle cells, and rabbit colonic muscle cells and stimulate Ca$^{2+}$ mobilization in cultured human colonic muscle cells (8, 15, 17, 31, 35, 39).

The signal-transduction mechanisms that mediate smooth muscle contraction by motilin have not been fully explored. Using rabbit duodenal muscle strips, Depoortere and Peeters (2) showed that motilin stimulated phosphoinositide (PI) hydrolysis to the same extent as carbachol and increased IP$_3$ formation. Motilin-induced PI hydrolysis was concentration dependent and paralleled motilin-induced contraction (2). In cultured, human colonic smooth muscle cells loaded with indomethacin-1, motilin caused an increase in cytosolic free Ca$^{2+}$ ([Ca$^{2+}$]$_i$), consistent with inositol 1,4,5-trisphosphate (IP$_3$)-dependent Ca$^{2+}$ release (35). Motilin-induced increase in [Ca$^{2+}$]$_i$ in the human medulloblastoma cell line TE671, which express motilin receptors, was retained in Ca$^{2+}$-free medium or the presence of the Ca$^{2+}$ channel blocker nifedipine (32).

Recent studies have shown that the contractile response to agonists consists of two phases: an initial transient phase mediated by IP$_3$-dependent Ca$^{2+}$ release and Ca$^{2+}$/calmodulin-dependent activation of myosin light chain (MLC) kinase (MLCK) leading to phosphorylation of MLC$_{20}$ and a sustained Ca$^{2+}$-independent phase mediated by inhibition of MLC phosphatase (21, 25). For Go$_{q}$/G$_{13}$-coupled receptors, inhibition of MLC phosphatase is mediated by Rho-dependent pathways involving Rho kinase-mediated phosphorylation of MYPT1, the regulatory subunit of MLC phosphatase, and/or PKC-dependent phosphorylation of CPI-17, an endogenous inhibitor.
of MLC phosphatase (25). Muscarinic M3 receptors and sphingosine-1 phosphate (SIP2) receptors engage both Rho-dependent pathways, whereas endothelin A (ET\_A) and lysophosphatidic acid (LPA\_3) receptors engage the MYPT1 and CPI-17 pathways, respectively (25, 39, 40).

In the present study, we have used freshly dispersed and cultured smooth muscle cells from the circular muscle layer of rabbit distal stomach and proximal small intestine to characterize the G protein-dependent signaling pathways mediating the initial and sustained phases of contraction and MLC20 phosphorylation. The results demonstrated that motilin initiates a transient Gq- and IP3-mediated calcium release and MLCK-dependent MLC20 phosphorylation and contraction and a sustained Gq\_11- and Gq13\_13-mediated cascade involving Rhokinase- and PKC-dependent inhibition of MLC phosphatase, resulting in sustained MLC20 phosphorylation and contraction.

MATERIALS AND METHODS

Preparation of dispersed and cultured gastric and intestinal smooth muscle cells. Smooth muscle cells were isolated from the circular muscle layer of rabbit distal stomach and proximal small intestine by sequential enzymatic digestion, filtration, and centrifugation as previously described (21, 24, 25). Muscle strips were incubated for 30 min at 31°C in 15 ml of HEPES medium containing 0.1% collagenase (type II) and 0.1% soybean trypsin inhibitor. The composition of the medium was (in mM) 120 NaCl, 4 KCl, 2.6 KH\_2PO\_4, 0.6 MgCl\_2, 25 HEPES, and 14 glucose, with 2.1% Eagle’s essential amino acid mixture. The partially digested tissues were washed with 100 ml of enzyme-free medium and reincubated for 30 min to allow spontaneous dispersion of muscle cells. Cells were harvested by filtration through 500 μm Nitex and centrifuged twice at 350 g for 10 min.

Functional studies were done on dispersed intestinal smooth muscle cells, whereas radioligand binding and transfection studies were done on gastric smooth muscle cells that can be more readily cultured. The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum until they attained confluence and were then passaged once for use in various studies.

Minigene construction and transfection into cultured gastric smooth muscle cells. Activation of specific G protein subunits was blocked by the expression of cDNA encoding the last COOH-terminal 11 amino acids of mouse G\_q\_1 and G\_q\_13 and human G\_o, as described previously (40). The cDNA sequences were amplified by PCR and verified by DNA sequencing. All G\_o minigene constructs used for transfection experiments were purified with an endotoxin-free maxi-prep kit (Qiagen). Cultured rabbit gastric smooth muscle cells were transiently transfected with minigene plasmid DNA using Effectene transfection reagent (Qiagen). Transfection efficiency was monitored by cotransfection of pGreen Lantern-1. Analysis by fluorescence microscopy showed that ~80% of the cells were transfected.

Expression of dominant negative GRK2 and RGS4 in cultured gastric smooth muscle cells. Dominant negative G protein-coupled receptor kinase 2 (GRK2) (K220R) or RGS4(N88S) was subcloned into the multiple cloning site (EcoRI) of the eukaryotic expression vector phevX, and a myc tag was incorporated into the NH\_2 terminus. Recombinant plasmid DNAs (2 μg each) were transiently transfected into smooth muscle cells in primary culture using Effectene transfection reagent for 48 h. The cells were cotransfected with 1 μg of pGreen Lantern-1 to monitor expression. Control cells were cotransfected with 2 μg of vector (pEXV) and 1 μg of pGreen Lantern-1 DNA. Transfection efficiency (~85%) was monitored microscopically by the expression of green fluorescent protein using FITC filters.

Identification of motilin-activated G proteins. G proteins activated by motilin were identified by an adaptation of method of Okamoto et al. (26), as described previously (23, 24). Dispersed intestinal muscle cells were homogenized in 20 mM HEPES (pH 7.4) containing 2 mM MgCl\_2, 1 mM EDTA, and 2 mM DTT, centrifuged at 30,000 g for 30 min and solubilized at 4°C in 20 mM HEPES (pH 7.4) buffer containing 0.5% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate. The solubilized membranes were incubated for 20 min at 37°C with 100 mM [35S]GTP\_S in 10 mM HEPES (pH 7.4) in the presence or absence of 1 μM motilin. After the reaction was stopped, the membranes were incubated for 2 h on ice in wells precoated with specific antibodies to Go\_q, Go\_11, Go\_12, Go\_13, and Gs\_13. The wells were washed with phosphate buffer containing 0.05% Tween 20, and radioactivity from each well was counted by liquid scintillation.

Characterization of motilin receptors by radioligand binding. Cultured gastric smooth muscle cells were redispersed and suspended in HEPES medium containing 1% BSA, amastatin (10 μM), phosphoramidon (1 μM), and bacitracin (0.7 mM). Triplet aliquots (0.3 ml) of cell suspension (10^6 cells/ml) were incubated for 15 min at 25°C with 125-I-labeled motilin (50 pM) alone or in the presence of unlabeled motilin (10 μM). Bound and free radioligand were separated by rapid filtration through 5-μm polycarbonate nucleopore filters followed by washing three times with HEPES medium. Nonspecific binding was measured as the amount of radioactivity associated with the muscle cells in the presence of unlabeled motilin (10 μM). Specific binding was calculated as the difference between total and nonspecific binding. Nonspecific binding was 20 ± 2% of total binding. Binding was also measured in cells expressing vector or dominant negative GRK2(K220R). The cells were first treated with 1 μM cold motilin for 20 min and washed rapidly for 5 min before specific 125-I-labeled motilin binding was determined.

Measurement of Ca\^{2+} release and [Ca\^{2+}], in muscle cells. Ca\^{2+} release was measured in dispersed intestinal muscle cells as described previously (23, 24). The cells were first permeabilized and suspended in a medium containing Ca\^{2+} (100 nM), 45Ca\^{2+} (10 μCi/ml), antimycin (10 μM), and an ATP regenerating system (1.5 mM ATP, 5 mM creatine phosphate, and 10 U/ml creatine kinase). Ca\^{2+} uptake was measured at intervals for 60 min when a steady state (2.53 ± 0.28 nmol/10^6 cells) was attained; IP3 or motilin was then added, and the reaction was terminated after 15 s. U-73122 or heparin was added 60 s before IP3 or motilin. The decrease in steady-state 45Ca\^{2+} cell content represented net Ca\^{2+} release expressed in nanomoles per 10^6 cells.

In some experiments, motilin-induced increase in [Ca\^{2+}], was measured by fluorescence in single smooth muscle cell loaded with fluorescent Ca\^{2+} dye fura 2 (24). Dispersed muscle cells were plated on coverslips for 12 h in DMEM. After being washed with PBS, the cells were loaded with 5 μM fura 2-AM for 1 h at room temperature. The cells were visualized through a ×40 objective (ZEISS; 0.9 NA) with a Zeiss Axioskop 2 plus upright fluorescence microscope and imaged with a setup consisting of a charge coupled device camera (Imago, TILL Photonics, Applied Scientific Instrumentation, Eugene, OR) attached to an image intensifier. The cells were alternately excited at 380 and 340 nm. The background and autofluorescence were corrected from images of a cell without the fura 2.

Assay for PI hydrolysis. PI hydrolysis was determined from the formation of total inositol phosphates using ion-exchange chromatography as described previously (23, 24). Cultured smooth muscle cells were labeled with myo-[3H]inositol (0.5 μCi/ml) for 24 h in inositol-free Dulbecco’s modified Eagle’s medium. The cultured cells were washed with PBS and treated with motilin (1 μM) for 30 s in 1 ml of 25 mM HEPES buffer (pH 7.4) containing (in mM) 115 NaCl, 5.8 KCl, 2.1 KH\_2PO\_4, 2 CaCl\_2, and 14 glucose. The reaction was terminated by the addition of 940 μl of chloroform-methanol-HCl (50:100:1). The samples were extracted with 310 μl chloroform and 340 μl of H\_2O, and then phases were separated by centrifugation at 1,000 g for 15 min. The upper aqeous phase was applied to DOWEX AG-1 column, and [3H]inositol phosphates were eluted with 0.8 M ammonium formamate-0.1 M formic acid. Radioactivity was deter-
mined by liquid scintillation and was expressed as counts per minute (cpm) per milligram of protein.

**Assay for Rho kinase activity.** Rho kinase activity was determined by immunokinase assay in cell extracts as described previously (25, 40). Rho kinase immunoprecipitates were washed twice with a phosphorylation buffer containing 10 mM MgCl₂ and 40 mM HEPES (pH 7.4) and then incubated for 5 min on ice with 5 μg of myelin basic protein. Rho kinase assay was initiated by the addition of 10 μCi of [γ-³²P]ATP (3,000 Ci/mmol) and 20 μM ATP, followed by incubation for 10 min at 37°C. ³²P-labeled myelin basic protein was absorbed onto phosphocellulose disks, and free radioactivity was removed by repeated washings with 75 mM phosphoric acid. The amount of radioactivity on the disks was measured by liquid scintillation.

**Immunoblot analysis of MLC20, MYPT1, and CPI-17 phosphoproteins.** Phosphorylation of MLC20, MYPT1, and CPI-17 was determined by immunoblot analysis with phosphospecific antibodies as described previously (25, 40). Muscle cells were treated with motilin for 30 s or 5 min in the presence or absence of various inhibitors and solubilized on ice in a medium containing 20 mM Tris·HCl (pH 8.0), 1 mM DTT, 100 mM NaCl, 0.5% SDS, 0.75% deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, and 100 μg/ml aprotinin. The lysate proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were incubated for 12 h with phosphospecific antibodies to MLC₂₀ (Ser¹⁹), MYPT₁ (Thr³⁸⁵), or CPI-17 (Thr¹⁸³) and then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. The bands were identified by enhanced chemiluminescence.

**Measurement of contraction in dispersed smooth muscle cells.** Contraction was determined in freshly dispersed gastric and intestinal circular muscle cells by scanning micrometry as described previously (21, 23, 24). A cell aliquot containing ~10⁶ cells/ml was treated with motilin in the presence or absence of various inhibitors; the reaction was terminated with 1% acrolein at a final concentration of 0.1%. The lengths of muscle cells treated with motilin were measured and compared with the lengths of untreated cells. Contractile response was expressed as percent decrease in mean cell length from control.

**Materials.** Motilin was obtained from Bachem (King of Prussia, PA), [³⁵S]GTPγS, myo-[³H]inositol, [³²P]ATP, and ⁴⁵Ca²⁺ were from New England Nuclear (Boston, MA); polyclonal antibodies to Gₛ, subunits, MLC₂₀, MYPT₁, and CPI-17 were from Santa Cruz Biotechnology (San Cruz, CA); U-73122, ML-9, Y27632, and bisindolylimidamide were from Calbiochem (San Diego, CA); pGreen

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**Fig. 1.** ¹²⁵I-labeled motilin binding to cultured muscle cells. A: muscle cells were incubated with ¹²⁵I-labeled motilin alone and in the presence of various concentrations of unlabeled motilin. Specific ¹²⁵I-labeled motilin binding was competitively inhibited by unlabeled motilin. B: ¹²⁵I-labeled motilin binding in cells expressing vector or dominant negative G protein-coupled receptor kinase 2 (GRK₂) (K220R) before and after treatment for 20 min with 1 μM motilin. Values are means ± SE of 8 experiments.

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**Fig. 2.** Time course and concentration dependence of motilin-induced contraction of dispersed smooth muscle cells. A: motilin (1 μM) was added to freshly dispersed intestinal circular smooth muscle cells for various intervals. Contraction consisted of a transient initial peak followed by a sustained contraction. B: motilin was added to dispersed smooth muscle cells at various concentrations for 30 s, and contraction was measured by scanning micrometry. Results are expressed as % decrease in cell length from control (mean control cell length: 121 ± 3 μm). Values are means ± SE of 4 experiments.
Lantern-1 and Lipofectamine Plus reagent were from Life Technologies GIBCO-BRL (Rockville, MD); and all other reagents were from Sigma.

RESULTS

Identification of motilin receptors. Motilin inhibited specific 125I-labeled motilin binding to cultured gastric smooth muscle in a concentration-dependent fashion with an IC50 of 0.7 ± 0.2 nM (Fig. 1A). The binding of 125I-labeled motilin to vector-transfected gastric muscle cells decreased by 87 ± 2% following exposure of the cells for 20 min to 1 μM motilin but decreased by only 21 ± 10% in cells expressing dominant negative GRK2(K220R) (Fig. 1B). The results implied that motilin receptors are internalized via a mechanism involving GRK2-mediated phosphorylation.

Treatment of freshly dispersed intestinal smooth muscle cells with motilin caused rapid contraction that attained a peak within 30 s followed by a sustained contraction of lower cells with motilin caused rapid contraction that attained a peak above basal level (1,488 ± 5% in cells overexpressing Gq minigene but not Gq11 minigene. Values are means ± SE of 4 experiments. **Significant inhibition from control response (P < 0.01).

Identification of G proteins coupled to motilin receptors in smooth muscle. Motilin caused an eightfold increase in the binding of [35S]GTPγS to Gq and a fivefold increase in the binding to Gq13 above basal level; there was no change in the binding of [35S]GTPγS to Goq, Gq11, Gq12, or Gq13 (Fig. 3). The pattern indicated that motilin receptors were selectively coupled to Gq and G13.

Gq-dependent stimulation of PI hydrolysis by motilin receptors. Motilin stimulated a fourfold increase in PI hydrolysis above basal level (1,488 ± 186 cpm/mg protein) in cultured gastric smooth muscle cells. PI hydrolysis decreased by 70 ± 5% in cells overexpressing Goq minigene but did not change significantly in cells overexpressing Gq11 minigene or Gq13 minigene (Fig. 4). PI hydrolysis decreased by 47 ± 3% in cells overexpressing wild-type RGS4 and increased by 43 ± 3% in cells overexpressing dominant negative RGS4(N88S) (Fig. 5A). The pattern implied that motilin-induced PI hydrolysis was mediated by Gq-dependent activation of PLC-β1 and attenuated by inactivation of Goq via RGS4.

Fig. 4. Motilin-induced phosphoinositide (PI) hydrolysis in cultured smooth muscle cells. Cultured gastric circular smooth muscle cells were prelabeled with myo-[3H]inositol, and PI hydrolysis in response to motilin was measured after 60 s by ion-exchange chromatography. Results are expressed as total [3H]inositol phosphate formation in counts per minute (cpm) per milligram of protein. PI hydrolysis was inhibited in cells expressing Goq, minigene but not Gq11 minigene. Values are means ± SE of 4 experiments. **Significant inhibition from control response (P < 0.01).

Fig. 5. Motilin-induced PI hydrolysis in cells expressing dominant negative RGS4(N88S) or GRK2(K220R). A: cultured gastric smooth muscle cells overexpressing RGS4(N88S), wild-type (WT) RGS4, or vector alone were pretreated for 20 min with 1 μM motilin, after which the PI hydrolysis in response to 1 μM motilin was determined. Values are means ± SE of 4 experiments. **Significant difference from response in vector-expressing cells (P < 0.01).

Fig. 3. Activation of G proteins by motilin in dispersed muscle cells. Membranes isolated from freshly dispersed intestinal smooth muscle cells were incubated for 20 min with [35S]GTPγS in the presence or absence of motilin (1 μM) in wells coated with various Go antibodies. Motilin caused selective activation of Goq and Gq13. Results are expressed as motilin-induced increase in [35S]GTPγS binding to specific Go isoforms. Values are means ± SE of 4 experiments. **Significant increase in the Go activation (P < 0.01).
After treatment of cultured gastric muscle cells for 20 min with 1 μM motilin, PI hydrolysis induced by motilin decreased by 72 ± 4% in cells expressing vector alone, by 86 ± 5% in cells overexpressing wild-type GRK2, and by 24 ± 7% in cells overexpressing dominant negative GRK2 (K220R) (P < 0.001 for the difference from cells expressing vector alone; Fig. 5B). The results provided further confirmation for the involvement of GRK2 in motilin-receptor desensitization.

Motilin-stimulated IP₃-dependent Ca²⁺ release. Motilin stimulated Ca²⁺ release (31 ± 2% decrease in steady-state ⁴⁵Ca²⁺ content in 15 s) in freshly dispersed permeabilized intestinal smooth muscle cells to the same extent as 1 μM IP₃ (32 ± 3%; Fig. 6). Ca²⁺ release was strongly inhibited by heparin, a blocker of IP₃ receptors, and U-73122, an inhibitor of PI hydrolysis (5 ± 1 and 3 ± 2% decrease in steady-state ⁴⁵Ca²⁺ content, respectively; P < 0.001 from control), implying that motilin induces a prompt Ca²⁺ release mediated by IP₃ (Fig. 5). Ca²⁺ release was accompanied by increase in cytosolic Ca²⁺ measured in fura 2-loaded single muscle cells (Fig. 6).

Pathways mediating motilin-induced initial smooth muscle contraction and MLC₂₀ phosphorylation. As shown in recent studies (23, 25, 40), the initial transient phase of muscle contraction (~1–2 min) induced by G protein-coupled receptor agonists in circular smooth muscle cells is Ca²⁺ dependent and involves stimulation of PI hydrolysis and IP₃-dependent Ca²⁺ release; Ca²⁺ binds to calmodulin and activates MLCK, resulting in phosphorylation of MLC₂₀ at Ser¹⁹, a prerequisite for contraction in smooth muscle. The initial contraction induced by motilin in intestinal circular muscle cells was virtually abolished by U-73122 and the MLCK inhibitor ML-9 but was not affected by the Rho kinase inhibitor Y27632 or the protein kinase (PKC) inhibitor bisindolylmaleimide (Fig. 7A). Treatment with 1 μM motilin after the addition of 1 μM ML-9, 1 μM Y27632, or 1 μM bisindolylmaleimide. MLC₂₀ phosphorylation was determined using phosphospecific Ser¹⁹ MLC₂₀ antibody. Equal amounts of protein (50 μg) were loaded and confirmed by Western blot analysis using MLC₂₀ antibody. Values are means ± SE of 3 experiments.
ment of the cells with the PLA2 inhibitor arachidonyltrifluoromethyl ketone for 10 min or with 400 ng/ml of pertussis toxin for 1 h had no effect on contraction induced by motilin (data not shown). Similarly, MLC20 phosphorylation during the initial phase of contraction was abolished by ML-9 but was not affected by Y27632 or bisindolylmaleimide (Fig. 8).

Similar results were obtained in dispersed gastric muscle cells, where initial contraction induced by motilin was virtually abolished by U-73122 and the MLCK inhibitor ML-9 but was not affected by Y27632 or bisindolylmaleimide (Fig. 9).

Fig. 9. Inhibition of motilin-induced initial and sustained muscle contraction in gastric circular muscle cells. Freshly dispersed gastric circular muscle cells were incubated with U-73122 (1 μM), ML-9 (10 μM), bisindolylmaleimide (1 μM), or Y27632 (1 μM) for 10 min and then treated with motilin (1 μM). Contraction was measured after 30 or 300 s. Muscle contraction was measured by scanning micrometry and expressed as % decrease in cell length from control (mean control cell length: 114 ± 4 μm). Values are means ± SE of 4 experiments.

Pathways mediating motilin-induced sustained muscle contraction and MLC20 phosphorylation. As shown for other Gq/G13-coupled agonists (25, 40), the sustained phase of contraction is Ca2⁺ independent and involves activation of RhoA leading to inhibition of MLC phosphatase and stimulation of MLC20 phosphorylation. Depending on the agonist, the steps leading to inhibition of the MLC phosphatase involve dual activation of Rho kinase and PLD/PKC by RhoA. Rho kinase phosphorylates MYPT1, the regulatory subunit of MLC phosphatase, whereas PKC phosphorylates CPI-17, an endogenous inhibitor of MLC phosphatase. Consistent with this notion, sustained contraction in dispersed intestinal circular muscle measured 5 min after treatment with motilin was partly inhibited by Y27632 and bisindolylmaleimide and abolished by a combination of both inhibitors but was not affected by U-73122 or ML-9 (Fig. 7B). Similarly, sustained MLC20 phosphorylation was inhibited by Y27632 and bisindolylmaleimide but was not affected by ML-9 (Fig. 8).

Similar data were obtained in dispersed gastric muscle cells, where sustained contraction was partly inhibited by Y27632 and bisindolylmaleimide and was abolished by a combination of both inhibitors but was not affected by U-73122 or ML-9 (Fig. 9).

Fig. 10. Motilin-induced activation of Rho kinase. Cultured gastric smooth muscle cells expressing Gαq, Gα11, or both Gαq and Gα11 minigenes were treated with motilin (1 μM) for 300 s, and Rho kinase activity was measured as described in MATERIALS AND METHODS. Results are expressed as cpm/mg protein. There is partial inhibition by each minigene and complete inhibition by both minigenes. Values are means ± SE of 4 experiments. **Significant inhibition from motilin-stimulated Rho kinase activity (P < 0.01).

Fig. 11. Motilin-induced phosphorylation of MYPT1 and CPI-17. Dispersed muscle cells were treated with motilin (1 μM) for 300 s. MYPT1 and CPI-17 phosphorylation was measured using phosphospecific Thr696 MYPT1 antibody and phosphospecific Thr38 CPI-17 antibody, respectively. Equal amounts of protein (50 μg) were loaded and confirmed by Western blot analysis using MYPT1 or CPI-17 antibody.


**G**q- and G13-dependent activation of Rho kinase by motilin. Depending on the agonist, RhoA is activated via G13 alone or via both G13 and Gq (9, 25, 40). Treatment of cultured gastric smooth muscle cells with motilin for 5 min caused a fourfold increase in Rho kinase activity above basal level (Fig. 10). Rho kinase activity was partly inhibited in cells expressing Goq minigene or Go13 minigene and was virtually abolished in cells coexpressing both minigenes (Fig. 10), implying participation of both G proteins in motilin-induced activation of RhoA.

**Motilin-induced phosphorylation of MYPT1 and CPI-17.** The pattern of sustained MLC20 phosphorylation and contraction by motilin suggested participation of Rho kinase-dependent phosphorylation of MYPT1 and PKC-dependent phosphorylation of CPI-17 in inhibition of MLC phosphatase. Treatment of cultured gastric smooth muscle cells with motilin for 5 min caused phosphorylation of CPI-17 at Thr38 and MYPT1 phosphorylation at Thr696 (Fig. 11A). CPI-17 phosphorylation was inhibited by bisindolylmaleimide but not by Y27632, whereas MYPT1 phosphorylation was inhibited by Y27632 but not by bisindolylmaleimide (Fig. 11B).

**DISCUSSION**

A number of previous studies have shown that motilin receptors are present in smooth muscle cells derived from various regions of the gut (stomach, small intestine, colon, gallbladder) in different species (human, dog, rat, and guinea pig) (2, 5, 7, 15, 30, 35). The receptors mediate contraction in circular smooth muscle by stimulating PI hydrolysis and IP3-dependent Ca2+ release and are capable of activating voltage-sensitive Ca2+ channels. Recent studies (25, 40) have shown, however, that agonist-induced contraction is a complex process that is only transiently dependent on Ca2+ mobilization.

In the present study, we provide a detailed analysis of the signaling pathways that mediate Ca2+-dependent and independent contraction by motilin receptors in gastric and intestinal smooth muscle cells. Motilin bound with high affinity (IC50 ~1 nM) to motilin receptors on smooth muscle cells. The receptors could be rapidly internalized and desensitized via a GRK2-mediated mechanism: expression of a dominant negative GRK2(K220R) blocked internalization as determined by the binding of 125I-labeled motilin to surface receptors and desensitization as determined by measurement of PI hydrolysis. Treatment of the cells with motilin induced a concentration-dependent contraction consisting of an initial transient phase followed by a sustained phase. Only the initial phase was Ca2+-dependent, reflecting Gq-mediated stimulation of PI hydrolysis and IP3-dependent Ca2+ release. The response was attenuated by wild-type RGS4 and augmented in cells overexpressing RGS4(N88S). Initial contraction and MLC20 phosphorylation were inhibited by the MLCK inhibitor ML-9 but were not affected by Rho kinase or PKC inhibitors. In this respect, motilin receptors conformed to a pattern observed with other Gq-coupled receptors in gastrointestinal smooth muscle, including muscarinic M3, CCK-A (23), endothelins ETα and ETβ, and neuropeptide Y.

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**Fig. 12. Pathways mediating initial and sustained contraction and MLC20 phosphorylation by motilin.** Initial contraction and MLC20 phosphorylation induced by motilin involve Gq-dependent PLC-β activation, IP3 generation and Ca2+ release, and Ca2+/calmodulin-dependent activation of MLC kinase. Sustained contraction and MLC20 phosphorylation involve Gα13 and Go13-dependent activation of Rho kinase, phosphorylation of MYPT1 and CPI-17, and inhibition of MLC phosphatase.
ET_b (9), 5-HT_2 (13), histamine H_1 (20), S1P_2 (40), neuropeptide Y/peptide YY Y_2, pancreatic polypeptide Y_4 (unpublished observation), UTP P2Y_2 (24), and LPA_3 (39) receptors, all of which stimulate PI hydrolysis by activating PLC-B1 via G_o_q.

During the sustained Ca^{2+}-independent phase, MLC_{20} phosphorylation and muscle contraction are mediated by G protein-dependent inhibition of MLC phosphatase. The pathways that lead to inhibition of MLC phosphatase vary with the agonist, but they usually involve phosphorylation of the regulatory subunit of MLC phosphatase via Rho kinase, phosphorylation of CPI-17, an endogenous inhibitor of MLC phosphatase via Rho kinase, or phosphorylation of both MYPT1 and CPI-17 (9, 25, 39, 40). G_q-coupled receptor agonists trigger a distinct pathway involving phosphorylation of both CPI-17 and MLC_{20} via integrin-linked kinase (ILK) (22). A role for ILK has been suggested also for the transient contraction induced in esophageal smooth muscle by phosphatase inhibitors (12). Inhibition of MLC phosphatase and stimulation of MLC_{20} phosphorylation by motilin receptors involves phosphorylation of both MYPT1 by Rho kinase and CPI-17 by PKC. The upstream pathway involves G_o_q and/or G_{13}-dependent activation of RhoA, which results in activation of both Rho kinase and PLD (25). Dephosphorylation of phosphatidic acid, the primary product of PLD activity, yields diacylglycerol, which, in turn, activates PKC. Sustained contraction and MLC_{20} phosphorylation by motilin were partially inhibited by the Rho kinase inhibitor Y27632 and the PKC inhibitor bisindolylmaleimide, and they were abolished by a combination of both inhibitors. In this respect, signaling by motilin receptors closely mimicked signaling by M_{3} and S1P_{2} receptors, where MLC_{20} phosphorylation results from coordinate inhibition of MLC phosphatase via MYPT1 and CPI-17 (25, 40). It differs from signaling by ETA receptors (9), which involves selective phosphorylation of MYPT1, or signaling by LPA_{3} receptors (39), which involves selective phosphorylation of CPI-17.

In summary, motilin receptors located on circular smooth muscle cells of the stomach and intestine are coupled to both G_o_q and G_{13}. The receptors are rapidly desensitized and internalized via a GRK2-dependent mechanism, and the initial G_o_q-mediated response (PI hydrolysis) is modulated by RGS4. Initial contraction and MLC_{20} phosphorylation by motilin is mediated by Ca^{2+}/calmodulin-dependent MLCK. In contrast, sustained contraction and MLC_{20} phosphorylation are mediated by a dual pathway involving G_{13}-mediated activation of RhoA, leading to phosphorylation of MYPT1 and CPI-17 by Rho kinase and PKC, respectively. Phosphorylation of both MYPT1 and CPI-17 caused cooperative inhibition of MLC phosphatase and resulted in sustained MLC_{20} phosphorylation and contraction. A model depicting the signaling pathways initiated by motilin receptors in gastrointestinal smooth muscle is depicted in Fig. 12.

GRANTS

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